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HEPATIC MICROSOMAL GLUCOSE-6-PHOSPHATASE OF NORMAL AND ALLOXAN-DIABETIC RATS

THERMOTROPIC EFFECTS ON KINETICS AND INTERACTION WITH DEOXYCHOLATE AND 1-ANILINO-8-NAPHTHALENE SULFONATE

VADDANAHALLY T. MADDAIAH, CRAIG L. STEMMER, SANDA CLEJAN and PLATON J. COLLIPP

Department of Pediatrics, Nassau County Medical Center and State University of New York, Stony Brook Health Sciences Center, East Meadow, NY 11554 (U.S.A.)

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Summary

Thermotropic effects on the kinetics of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) activity of hepatic microsomes from normal and alloxan-diabetic rat liver were investigated by determining V, K_m and K_i (substrate inhibition) values. Influence of deoxycholate (0.1%) and 1-anilino-8-naphthalene sulfonate (2.5 mM) on the kinetics was also evaluated.

1. Substrate inhibition occurred at 0.06 M for the enzyme from normal rats and at 0.02—0.025 M for the enzyme from diabetic rats.

2. The enzyme from diabetic rats showed a transition that extended between 22.7 and 27°C in the Arrhenius plot (log V vs. T^{-1}) instead of at 19.5°C.

3. Deoxycholate increased the V value of both enzymes without affecting substrate inhibition at all the temperatures but did not completely abolish the transition in the Arrhenius plot of the enzyme from diabetic rats.

4. 1-Anilino-8-naphthalene sulfonate eliminated substrate inhibition and activated the enzyme of normal rats above 27.5°C by increasing both V and K_m values. Below this temperature, the enzyme showed biphasic or allosteric kinetics. At low substrate concentrations the enzyme was inhibited competitively, whereas at high substrate concentrations it was activated as both V and K_m values were increased. The enzyme from diabetic rats, on the other hand, was activated at all the temperatures and exhibited linear kinetics.

5. Binding of 1-anilino-8-naphthalene sulfonate to the microsomal fraction increased with decreasing temperature as revealed by the increase of relative fluorescence. The microsomal fraction of diabetic rats showed a more

anomalous fluorescence response between 13—18°C.

6. Enthalpy changes for glucose 6-phosphate binding to the inhibition site were slightly larger than binding to the active site. Calculated entropies of activation for transition state complex of glucose-6-phosphatase reaction were fairly large and negative. The free energy of activation (28—30 kcal/mol) was independent of temperature and experimental conditions.

7. In the microsomal fraction (total as well as rough), phospholipid content and fatty acid unsaturation index of phospholipids were decreased after diabetes. The level of free cholesterol remained unchanged but the molar ratio of cholesterol to phospholipid increased. The different thermal response and 1-anilino-8-naphthalene sulfonate interaction of the enzyme from diabetic rat liver could be ascribed to the altered lipid environment of the enzyme on the endoplasmic reticulum membrane.

Introduction

The dynamic state of bilayer lipids is now recognized as playing an important role in the regulation of the activity of membrane associated enzymes [1]. Hepatic glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9), which plays a unique role in glucose homeostasis [2], exists mostly on the endoplasmic reticulum membrane [3] but recently it has also been found on the nuclear membrane [4]. It is an integral component of the endoplasmic reticulum membrane since the enzyme protein is susceptible to proteolysis only in the presence of 0.05% deoxycholate [5]. The enzyme is responsive to nutritional and hormonal stimuli and the activity is increased in diabetes [2]. The enzyme, in addition to phosphatase, also shows pyrophosphatase and phosphotransferase activities [6]. It has also been proposed that glucose-6-phosphatase is a two component system consisting of a specific carrier for glucose 6-phosphate on the cytoplasmic side and a nonspecific phosphatase at the luminal side of the endoplasmic reticulum membrane [7].

Phospholipid requirement for glucose-6-phosphatase activity has been investigated [8—10]. Thermotropic phase changes of membrane lipids influence the enzyme, since an Arrhenius plot of activity measured at one substrate concentration [11,12] shows a major transition at 20°C. However, it has been recently proposed [13] that generation of Arrhenius plots by measuring enzyme activities at one concentration of the substrate could lead to erroneous conclusions. We have been studying glucose-6-phosphatase with a view to understanding the relationship between membrane structure and enzyme activity [14—16]. In these studies, effects of different reagents and 1-anilino-8-naphthalene sulfonate on enzyme activity of freeze-dried and resuspended microsomes were investigated. In order to generate Arrhenius and Van't Hoff plots we have now measured the kinetics of glucose-6-phosphatase from once freeze-thawed microsomes of normal and diabetic rat liver in the presence and absence of deoxycholate and anilinonaphthalene sulfonate. The enzyme from diabetic rats showed a different thermal response which may be ascribed to the altered lipid environment of the enzyme on the endoplasmic reticulum membrane.

Materials and Methods

Normal male Sprague-Dawley rats (200—250 g) were maintained on Purina chow ad libitum. The animals were made diabetic by one intraperitoneal injection of an aqueous solution of alloxan (100 mg/kg body weight). Urinary glucose was tested twice daily with Labstix (Ames) and only those with a color of $3—4^+$, which corresponds to 1—2 mg% urinary glucose and 300 mg% blood glucose, were considered as diabetic. Rats were killed and liver was removed into ice-cold 0.25 M sucrose. Liver was chopped and washed three times with ice-cold sucrose. A 10% homogenate of liver (w/v) was prepared in 0.25 M sucrose and centrifuged at $12\,000 \times g$ for 10 min. The supernate was centrifuged at $105\,000 \times g$ for 1 h and the residue was washed once with 0.25 M sucrose. The residue was suspended in one-fourth of the original volume of 0.25 M sucrose by hand homogenization and stored in small aliquots at —30°C. Each aliquot was thawed in ice-cold water and used only once for kinetic measurement. Rough microsomes were prepared from a 25% homogenate of liver as described by Dallner [17].

Estimation of glucose-6-phosphatase activity

Glucose-6-phosphatase activity in duplicate tubes was measured at pH 6.5 in 0.06 M maleate buffer as described [15—16]. The pH values of glucose 6-phosphate substrate and other solutions were adjusted to 6.5. Required temperature was maintained in an ambient water bath with shaking (Blue M, Blue Island, IL, U.S.A.). Deoxycholate and anilinonaphthalene sulfonate solutions were added to the enzyme suspension in the buffer at 0°C and left to stand for 5 min. The tubes were allowed to stand for 3 min at the assay temperature before starting the reaction by the addition of glucose 6-phosphate. Enzyme activity is usually expressed as mmol of P liberated/min per mg protein. Protein was assayed by the method of Lowry et al. [18] using bovine serum albumin as the standard. Anilinonaphthalene sulfonate was recrystallized once in water and its solution concentration was determined at 350 nm using $6 \cdot 10^3$ [19] as molar absorptivity. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Preliminary kinetic experiments indicated substrate inhibition and, therefore, concentration of the substrate glucose 6-phosphate (pH 6.5) was varied from 0.15 to 0.005 M in the assay. The phosphate value at each substrate concentration was corrected for its corresponding zero-time value [15—16]. Since variation of activity with substrate concentration was non-linear the data were analyzed by Eadie-Hofstee plots. Kinetic parameters were calculated by linear regression analysis and best line-fitting of the data from at least two experiments using two different enzyme preparations.

The energy of activation, E_a, was calculated from the Arrhenius plot, the slope being $-E_a/R$. The free energy of activation, ΔG^* was determined from the followed relationship of transition state theory,

$$k_r = (\mathrm{k}T/h)\ 3 - \Delta G^*/RT;\ k_r = V/(\text{total enzyme})$$

$$= \text{units } s^{-1} \cdot mg^{-1} \text{ per units } mg^{-1}$$

where k_r is the rate constant, and k and h are Boltzman's and Planck's con-

stants, respectively. Maximum velocity, V, obtained by extrapolation of the linear portions of the Eadie-Hofstee plot at non-inhibiting substrate concentrations and expressed as mol P/s per mg protein was taken as equivalent to k_r. The entropy of activation, ΔS^*, was calculated from the thermodynamic relationship,

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \text{ and } \Delta H^* = E_a - RT$$

where ΔH^* is the enthalpy of activation.

Estimation of lipids

Freshly prepared microsomal fraction was extracted twice with chloroform/methanol (2 : 1, v/v) (5 ml solvent/mg protein) under nitrogen. The extract was filtered and the pooled filtrate was washed with 0.2 vol. of 0.9% NaCl according to Folch et al. [20]. The lower phase was collected and evaporated. The residue was dissolved in chloroform and fractionated chromatographically into neutral and polar phospholipids on a column of silicic acid (100 mesh, 1 g/30 mg total lipids). Neutral lipids were first eluted with 20 ml chloroform and phospholipids with 20 ml methanol. Neutral lipids were further separated by TLC on silica gel G plates which were developed at room tempetature with a mixture of benzene/ethyl acetate (5 : 1, v/v). Solutions of free cholesterol (10—30 μg/ml) were used as standards on the silica gel plates. Free cholesterol spots on the plates were scraped and extracted into chloroform/methanol (2 : 1, v/v) at 45°C for 1 h. The mixture was centrifuged and after evaporating chloroform, the free cholesterol was estimated by the method of Zlatkis and Zak [21]. Phospholipids were separated by TLC on silica gel either by single migration using a solvent mixture of chloroform/methanol/water (72 : 25 : 4, v/v) or by a two-dimensional migration involving first chloroform/methanol/water/28% ammonia (130 : 70 : 8 : 0.5, v/v) followed by chloroform/acetone/methanol/acetic acid/water (100 : 40 : 20 : 20 : 10, v/v) according to Parsons and Patton [22]. The chromatographic plates were exposed to iodine vapor. The individual phospholipids were identified with synthetic phospholipid standards (Sigma Chemical Co., St. Louis, MO). The spots were scraped off and eluted by washing with chloroform/methanol (2 : 1, v/v). Quantitative analysis of phospholipid fractions was then effected by phosphorus determination according to Bartlett [23]. The fatty acids were analyzed by gas-liquid chromatography after conversion to methyl esters by refluxing for 2 h with 6 ml of a solution of 1 ml H_2SO_4 conc./61.5 ml methanol/123 ml benzene. Identification of methyl esters was carried out with a Hewlett-Packard 5710A gas chromatograph equipped with dual column and dual flame ionization detectors. The stainless column (6 ft. × 1/8 inch inner dimater) was packed with 80—100 mesh chromosorb (acid washed) coated with 20% (w/w) ethylene glycol succinate/2% phosphoric acid (Supelco, Inc., Bellfonte, PA, U.S.A.). Routine analyses were performed isothermally at 185°C with a carrier gas-flow rate 30 ml/min. Peak identifications were based on relative retention times relative to methyl stearate and comparison with a known mixture of standard methyl ester (Sigma Chemical Co., St. Louis, MO). Relative peak areas were measured by a Hewlett-Packard 3380A electronic integrator. The unsaturation index was calculated as the sum of the mol fraction multiplied by the number of unsaturated bonds in each acid.

Results

Effects of deoxycholate on glucose-6-phosphatase activity

Glucose-6-phosphatase activity at varying concentrations of the substrate was measured at a number of temperatures ranging from 4.9—34.9°C. Small changes in pH due to variations in temperature may have negligible influence on the activity since the phosphatase has a broad range of pH optimum [24]. Eadie-Hofstee plots of untreated glucose-6-phosphatase activity of normal rat liver measured at 33.2 and 9.5°C are shown in Figs. 1 and 2. As can be seen, there is inhibition of the activity of untreated microsomes beyond 0.06 M glucose 6-phosphate. Values for V and K_m were obtained from the extrapolated linear part of the plot at low substrate concentrations. Substrate inhibition (K_i) was obtained from the slope of the best-fitting line for the plot at high substrate concentrations. Addition of 0.1% deoxycholate to the assay medium did not change substrate inhibition but increased the activity (data not shown). The enzyme from diabetic rat liver has higher activity and the Eadie-Hofstee plots are similar to those of Figs. 1 and 2. The Arrhenius plot (data not shown) of the activity of untreated microsomes from normal rat showed a break or transition at 19.5°C which is similar to that reported first by Eletr et al. [11] and later by Grinna [12] for the activity of fresh microsomes. Generation of Arrhenius plots using the activity at one inhibiting concentration of glucose 6-phosphate also gives the transition at about 19.0°C. The energy of activation (E_a) is small and may be balanced by the relatively large but negative entropy of activation, ΔS^* (Table I). The presence of 0.1% deoxycholate in the assay

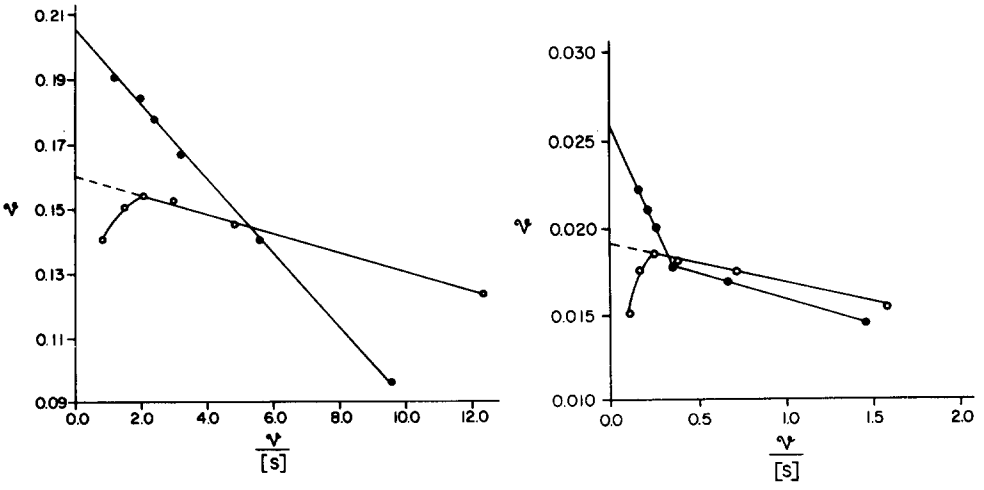

Fig. 1. Eadie-Hofstee plot of hepatic microsomal glucose-6-phosphatase activity of normal rat liver at 33.2°C in the presence (●———●) and absence (○———○) of 2.5 mM 1-anilino-8-naphthalene sulfonate. V was obtained by extrapolating the linear part (- - - - -).

Fig. 2. Eadie-Hofstee plot of hepatic microsomal glucose-6-phosphatase activity of normal rat liver at 9.5°C in the presence (●———●) and absence (○———○) of 2.5 mM 1-anilino-8-naphthalene sulfonate. V is obtained by extrapolating the linear part (- - - - -).

TABLE I

EFFECTS OF DEOXYCHOLATE AND 1-ANILINO-8-NAPHTHALENE-SULFONATE ON TERMODYNAMIC PARAMETERS OF ACTIVATION OF GLUCOSE-6-PHOSPHATASE OF NORMAL (N) AND ALLOXAN-DIABETIC (D) RATS

Transition temperatures (T_t) and energies of activation (E_a) were obtained from the Arrhenius plots of glucose-6-phosphatase activity in the temperature range of 4.9—34.9°C. The free energies of activation (ΔG^*) were calculated according to transition state theory. The maximum velocity (V), obtained by extrapolation of the linear portions of the Eadie-Hofstee plot, was expressed as mol P liberated/s per mg protein and taken as the rate constant. The entropies of activation (ΔS^*) were calculated from the relationship $\Delta S^* = (\Delta H^* = \Delta G^*) \cdot T^{-1}$ where the enthalpy of activation, $\Delta H^* = E_a - RT$. Free energy of activation was 28—30 kcal/mol which did not vary with experimental conditions.

Addition	T_t (0°C)		E_a (kcal/mol)				ΔS^* (cal/mol per degree)			
	N	D	Above T_t		Below T_t		31.0°C		9.5°C	
			N	D	N	D	N	D	N	D
None	19.5	27.0	13.18	7.27	15.16	22.66	—57.02	—74.85	—50.21	—47.83
	—	22.7		22.66	—	15.33				
Deoxycholate (0.1%)	—	27.0	12.0	10.02	—	—	—60.49	—65.44	—63.67	—62.59
		17.6		—		10.88				
1-Anilino-8-naphthalene sulfonate (2.5 mM)	27.0	25.5	18.87	10.51	14.18	21.22	—38.11	—59.69	—52.75	—52.59
		21.5		21.22	15.29 *	13.78			—49.73 *	
1-Anilino-8-naphthalene sulfonate (2.5 mM) + deoxycholate (0.1%)	25.0	27.5	12.04	4.38	10.98	17.42	—61.62	—80.00	—60.07	—63.51
		25.0		17.42	13.30 *	10.80			—56.53 *	

* These values were calculated from linear low V plots below the transition temperature in the presence of 1-anilino-8-naphthalene sulfonate.

medium increased the V value at all temperatures, but abolished the transition at 19.5°C. The Arrhenius plot of untreated enzyme from diabetic rats (Fig. 3) shows two major inflections, one at 27.0°C and another at about 22.7°C. The transition temperature of 22.7°C is obtained from the point of intersection of the two extrapolated lines. Energy of activation (E_a) between 27.0 and 22.7°C is larger than that of either the high temperature region or the low temperature region (Table I). Entropies of activation (ΔS^*) are negative and are almost identical to those of the enzyme from normal rat liver at 9.5°C (Table I). The presence of deoxycholate increases the V value at all the temperatures but does not completely abolish the transition as it did with the enzyme from normal rat liver. Thermodynamic parameters remain almost unchanged (Table I).

Effects of temperature on substrate affinity (K_m) and substrate inhibition (K_i) were evaluated from van't Hoff plots (Fig. 4) as shown for the enzyme from diabetic rats. There is no transition in the pK_m plot for the enzyme of normal rat but there are two inflections, at 14.0 and 22.4°C, for the enzyme from diabetic rats. There is a transition at 25.0°C in the pK_i plot for the enzyme from normal rat liver and the addition of deoxycholate does not affect it. Enthalpy changes for binding of glucose 6-phosphate to the inhibition site, derived from pK_i plots, are generally more negative than for binding to the active site (Table II).

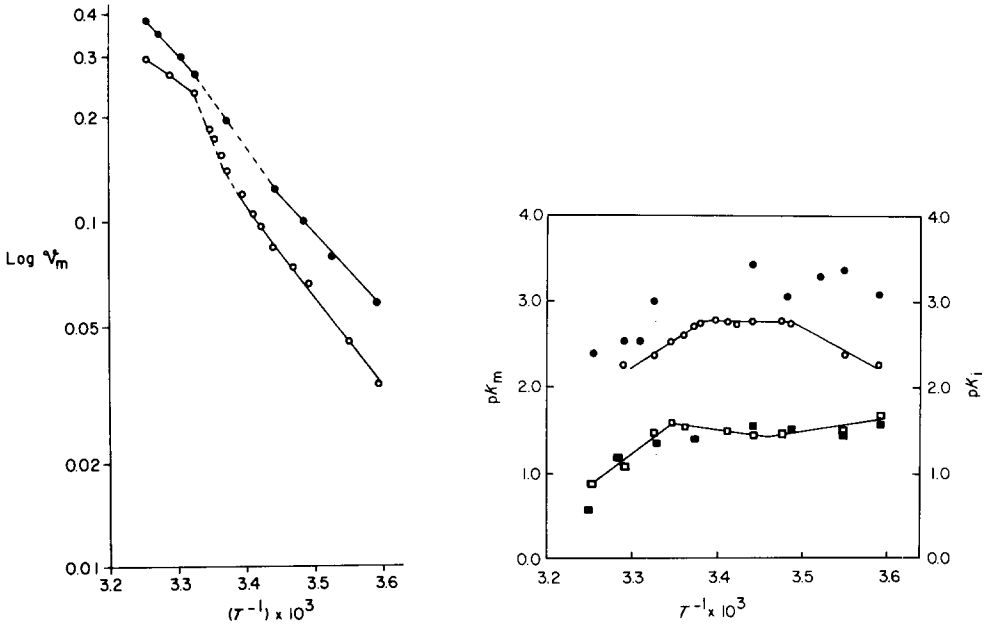

Fig. 3. Arrhenius plots of microsomal glucose-6-phosphatase activity of alloxan-diabetic rat liver. Additions: ○———○, none; ●———●, 0.1% deoxycholate. V values were obtained by extrapolation of Eadie-Hofstee plots.

Fig. 4. Van't Hoff plots of microsomal glucose-6-phosphatase activity of alloxan-diabetic rat liver. Additions: pK_m, ○———○, none; ●———●, 0.1% deoxycholate; pK_i, □———□, none; ■———■, 0.1% deoxycholate. K_m and K_i values were calculated from the slopes of the corresponding linear portions of Eadie-Hofstee plots.

TABLE II

EFFECTS OF DEOXYCHOLATE AND 1-ANILINO-8-NAPHTHALENE SULFONATE ON ENTHALPY CHANGES (ΔH^*) OF BINDING OF GLUCOSE 6-PHOSPHATE TO HEPATIC GLUCOSE-6-PHOSPHATASE OF NORMAL (N) AND ALLOXAN-DIABETIC (D) RATS

Additions	Binding to active site *						Binding to inhibition site **					
	T_t (°C)		ΔH^* (kcal)				T_t (°C)		ΔH^* (kcal)			
	N	D	Above T_t		Below T_t		N	D	Above T_t		Below T_t	
			N	D	N	D			N	D	N	D
None	—	22.4	—3.3	—18.5	—	+2.1	25.0	25.9	—27.7	—16.3	—3.1	+2.5
	—	14.0	—	+2.1	—	+22.4	—	16.1		+2.5	—	—1.3
Deoxycholate (0.1%)	—	—***	—12.6	—***	—	—	25.0	25.9	—25.6	—16.3	—2.9	+2.5
							16.1			+2.5		—1.3
1-Anilino-8-naphthalene sulfonate (2.5 mM)	23.0	20.5	—30.4	—25.1	+4.9	+0.9		—		—		—
1-Anilino-8-naphthalene sulfonate (25 mM) + deoxycholate (0.1%)	—	25.0	—4.7	—13.8		+2.1		—		—		—

* Transition temperature (T_t) and enthalpy changes (ΔH^*) were obtained from the plots of pK_m vs. T^{-1}.

** Transition temperatures (T_t) and enthalpy changes were obtained from the plots of pK_i vs. T^{-1}.

*** No attempt was made to derive the transition temperature and enthalpy change due to low correlation coefficients for best-fitting lines.

Effects of anilinonaphthalene sulfonate on glucose-6-phosphatase activity

Kinetic plots of glucose-6-phosphatase activity of normal rat liver measured in the presence and absence of 2.5 mM anilinonaphthalene sulfonate at 33.2 and 9.5°C are shown in Figs. 1 and 2. These studies at 33.0 and 9.5°C were repeated with fresh unfrozen microsomal preparations of normal and diabetic rat livers and the results (data not shown) were identical to those presented here. Anilinonaphthalene sulfonate completely eliminated the substrate inhibition at both temperatures. As a result, at 33.2°C, the kinetics are linear. V and K_m values (apparent) are both increased resulting in activation of the enzyme at all temperatures studied above 27.5°C. Below this temperature, as can be seen in Fig. 2 (9.5°C), the kinetics in the presence of 2.5 mM anilinonaphthalene sulfonate are biphasic or allosteric. At substrate concentrations lower than 0.05 M there is inhibition which is competitive with anilinonaphthalene sulfonate. At substrate concentrations above 0.05 M the activity increases linearly resulting in activation. Therefore, at these temperatures, two sets of kinetic parameters (V and K_m) could be generated. Eadie-Hofstee plots (data not shown) for the enzyme from diabetic rat liver were linear at all the temperatures studied as the presence of anilinonaphthalene sulfonate caused activation at all the substrate concentrations. Therefore, the major difference of the enzyme from diabetic rats is the absence of biphasic or allosteric kinetics at temperatures lower than 27.5°C. The Arrhenius plot (Fig. 5) for the enzyme

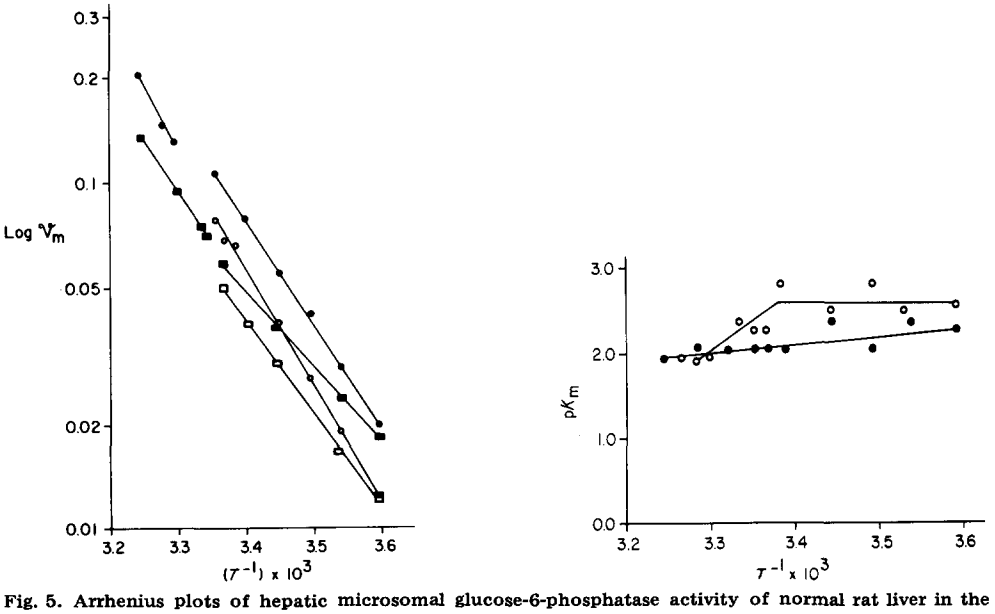

Fig. 5. Arrhenius plots of hepatic microsomal glucose-6-phosphatase activity of normal rat liver in the presence of 2.5 mM 1-anilino-8-naphthalene sulfonate (●———●, ○———○, low V) and 2.5 mM 1-anilino-8-naphthalene sulfonate + 0.1% deoxycholate (■———■, □———□, low V). V_m values were obtained by extrapolation of the corresponding linear portions of Eadie-Hofstee plots (see Text for details).

Fig. 6. Van't Hoff plots of hepatic microsomal glucose-6-phosphatase activity of normal rat liver in the presence of 2.5 mM 1-anilino-8-naphthalene sulfonate (○———○) and in the presence of 2.5 mM 1-anilino-8-naphthalene sulfonate + 0.1% deoxycholate (●———●). K_m values were calculated from the slopes of the corresponding linear portions of Eadie-Hofstee plots.

from normal rats with two sets of V values shows a clear transition at 27.5°C. The Arrhenius plot which includes high V values was similar to that of deoxycholate-treated microsomes, whereas the plot of low V values below 27.5°C was superimposable on the plot of untreated microsomes. Changes in the activation energies are slight. The extent of activation by anilinonaphthalene sulfonate is similar to that of deoxycholate but for the presence of a transition at 27.5°C (Table I). The Arrhenius plot (data not shown) for the enzyme of diabetic rat liver showed that the V value is increased at all temperatures. There was a clear transition in the activation energy at 21.5°C above which the plot was non-linear. By the method of 'best-fit', another transition at about 25.5°C could be detected. The activation parameters do not differ from those of the untreated enzyme (Table I).

In the evaluation of the effects of temperature on the apparent K_m of glucose-6-phosphatase of normal rats in the presence of 2.5 mM anilinonaphthalene sulfonate (Fig. 6) only high K_m values (<27.5°C) were included since it showed maximum deflection or transition at about 23.0°C. The transition is similar to that of pK_i vs. T^{-1} plot with similar enthalpies of binding shown by the untreated enzyme (Table II). If low K_m values are used the plot will be similar to that of untreated microsomes. It is important to note here that at temperatures below 27.5°C, the presence of anilinonaphthalene sulfonate gives rise to two active enzyme-substrate binding sites. One of the sites has a high affinity for glucose 6-phosphate and shows competition for binding with anilinonaphthalene sulfonate. The other has a low affinity for glucose 6-phosphate and is probably identical to the inhibitory substrate binding site in the absence of anilinonaphthalene sulfonate. The pK_m vs. T^{-1} plot (data not shown) for the enzyme from diabetic rats showed a transition at 20.5°C.

Effects of temperature on anilinonaphthalene sulfonate fluorescence

The appearance of allosteric kinetics at low temperatures for the enzyme of normal rats could be due to increased binding of anilinonaphthalene sulfonate to microsomes. Therefore, we measured the effect of temperature on the relative fluorescence when anilinonaphthalene sulfonate (10 μM) was added to microsomes of normal and diabetic rats. As shown in the semi-log plot of relative fluorescence vs. T^{-1} (Fig. 7) fluorescence increased almost linearly with decreasing temperature but the response was anomolous, which was more pronounced in the region of 13—18°C for the microsomes from diabetic rats than those from normal rats. This temperature range is below the transition in activation energy and is in the transition region of binding enthalpies of the untreated enzyme (Table II). Recently, Zierler and Rogus [25] analyzed the effects of temperature on fluorescence characteristics of anilinonaphthalene sulfonate and concluded that binding increases, particularly to the protein component of the sarcolemma vesicles, as the temperature is decreased.

Effects of deoxycholate and anilinonaphthalene sulfonate on glucose-6-phosphatase activity

Addition of both deoxycholate (0.1%) and anilinonaphthalene sulfonate (2.5 mM) decreased the glucose-6-phosphatase activity of normal rats even when the activity was compared with that of untreated microsomes and the kinetics were

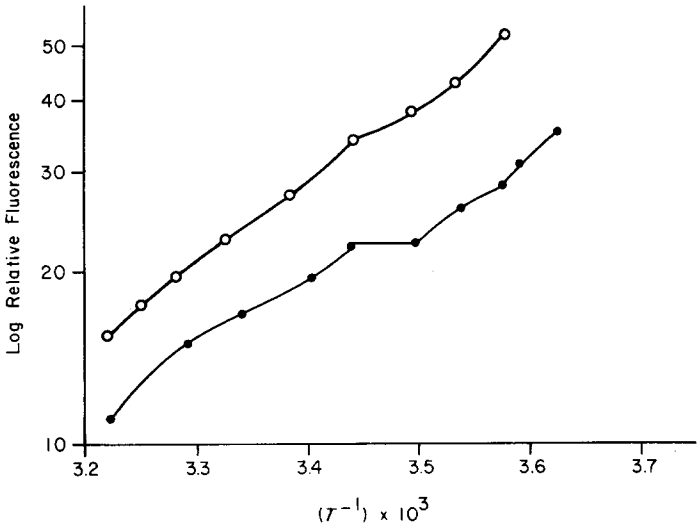

Fig. 7. Effect of temperature on relative fluorescence (excitation at 375 nm and emission at 475 nm) of 1-anilino-8-naphthalene sulfonate (10 μM) and microsomal fraction of normal (○———○) rat liver (1 mg protein/ml) and alloxan-diabetic (●———●) rat liver (1.0 mg protein/ml).

linear up to 25.5°C, below which the effects were allosterically similar to those seen in the presence of only anilinonaphthalene sulfonate (data not shown). Therefore, below this temperature two sets of kinetic parameters are generated by extrapolation of the two linear portions of the curves. In the Arrhenius plot (Fig. 5) there is a transition at 25.0°C below which two plots (high and low V) are shown. The thermodynamic parameters of activation (Table I) are similar to those in the presence of only deoxycholate. The van't Hoff plot that includes only high K_m values (below 25.0°C) did not shown any clear discontinuity. The activity of diabetic rats was inhibited when compared to that in the presence of either only deoxycholate or anilinonaphthalene sulfonate, but, was almost unchanged when compared to that of the untreated enzyme. The Arrhenius plot (data not shown) showed a major transition at 25.0°C above which it was nonlinear. Another transition at about 27.5°C could be detected in the nonlinear part. The energy of activation (Table I) above 27.5°C is the lowest (4.38 kcal/mol). The other activation parameters are similar to those of the untreated enzyme. In the plot of apparent pK_m vs. T^{-1} (data not shown) there was a transition at 25.0°C below which the enthalpy change was negligible (Table I). Substrate inhibition (K_i) was almost unchanged at the temperatures studied.

Lipid and fatty acid composition of hepatic microsomes

Since some of the differences of thermotropic effects and interaction with anilinonaphthalene sulfonate could be due to an altered lipid environment of the enzyme in diabetes, the phospholipid and cholesterol content as well as the distribution of main phospholipid classes and the fatty acid distribution in phospholipids were estimated. Total phospholipid (μg P/mg protein) is decreased significantly in the diabetic rats and the decrease is larger in rough microsomes than in total microsomes (Table III). A decrease in lipid phosphate

TABLE III

PHOSPHOLIPID AND FREE CHOLESTEROL CONTENT OF HEPATIC MICROSOMAL FRACTIONS FROM NORMAL AND ALLOXAN-DIABETIC RATS

Methods of estimation are described in the text and the values are given as means ± S.D. Numbers in parenthesis represent the number of samples and each sample is from a different animal.

	Total microsomes		Rough microsomes	
	Normal (5)	Diabetic (3)	Normal (4)	Diabetic (4)
Total phospholipids * (μg/mg protein)	177.8 ± 11.3	134.0 ± 0.6	288.3 ± 13.3	157.2 ± 12.0
Free cholesterol (μg/mg protein)	18.7 ± 1.2	18.9 ± 0.7	9.6 ± 0.6	8.9 ± 0.2
Cholesterol: total phospholipid (molar ratio)	0.20	0.27	0.08	0.14

* The average molecular weight of phospholipid is taken as 930. Distribution (% total phospholipids) of phosphatidylcholine, phosphatidylinositol, phosphatidylethanol amine + phosphatidylserine, sphingomyelin, and cardiolipin are:

normal: 59.0 ± 0.8, 6.6 ± 0.7, 29.5 ± 0.4, 3.6 ± 0.3 and 1.0 ± 0.1,
diabetic: 61.2 ± 1.2, 5.6 ± 0.3, 28.1 ± 1.5, 4.2 ± 0.1 and 0.9 ± 0.1, respectively.

Two-dimensional TLC of total microsomal fractions from one normal and one diabetic rat gave about 3.8% for phosphatidylserine.

concentration of whole liver has been reported in diabetic rats [26]. Individual phospholipids in total microsomes, expressed as percent of total, are unchanged. Therefore, the decrease may be uniformly distributed in all the phospholipid fractions of diabetic rats. Free cholesterol content remains unchanged after diabetes, but, most notably the percent molar ratio of cholesterol to phospholipid increases from 20 to 27 in whole microsomes and from 8 to 14 in rough microsomes (Table III). Phospholipid and cholesterol content of rough microsomes from normal rat liver are similar to those reported by Fleischer and Kervina [27].

The fatty acid distribution in total and individual phospholipids of the total microsomal fractions of normal and diabetic rat liver are shown as percent of the total fatty acids (Table IV). Palmitic (16:0) and stearic (18:0) acids constitute almost 50% of the total fatty acids. The phosphatidylinositol contains an even larger percentage of the two acids. Among the unsaturated acids, arachidonic (20:4) makes the largest contribution towards the unsaturation index, which is significantly decreased in the total phospholipid fractions ($P < 0.05$) and in phosphatidylethanolamine + phosphatidylserine fractions ($P < 0.01$) in the diabetic rats. Also, docosahexaenoic acid (22:6) is decreased in the total phospholipid ($P < 0.01$) and phosphatidylethanolamine + phosphatidylserine fractions ($P < 0.02$).

Discussion

Generally, the influence of thermotropic phase transition of lipids on membrane-associated enzymes becomes recognizable when a break or transition appears in an Arrhenius plot. The Arrhenius plot of glucose-6-phosphatase

TABLE IV

FATTY ACID DISTRIBUTION IN PHOSPHOLIPIDS AND IN SOME INDIVIDUAL FRACTIONS OF PHOSPHOLIPIDS OF HEPATIC MICROSOMES FROM NORMAL (N) AND ALLOXAN-DIABETIC (D) RATS

The preparation of microsomes, the extraction of lipids, the separation of phospholipids and the analysis of fatty acids are described in Methods. The results of fatty acids are expressed as mol fraction percent. Data represent mean values ± S.D. Numbers in parenthesis represent the number of samples and each sample is from a different animal. Minor phospholipid fractions are: sphingomyelin (3.6 ± 0.3% in N and 4.2 ± 0.1% in D rats) and cardiolipin(1.0 ± 0.1% in N and 0.9 ± 0.1% in D).

Fatty acid	Total phospholipids		Phosphatidylcholine		Phosphatidylinositol		Phosphatidylethanolamine + phosphatidylserine	
	N (5)	D (6)	N (4)	D (3)	N (4)	D (3)	N (4)	D (3)
14:0	0.16 ± 0.2 [c]	0.23 ± 0.03	0.8 ± 0.07	0.7 ± 0.02	2.6 ± 0.2	2.5 ± 0.3	—	1.7 ± 0.2
16:0	19.4 ± 1.5	20.0 ± 0.9	24.1 ± 0.3	24.2 ± 0.4	19.1 ± 1.0	18.8 ± 1.1	22.8 ± 0.2	23.6 ± 0.3
16:1	0.26 ± 0.04	0.24 ± 0.02	3.6 ± 0.5	3.6 ± 0.1	1.1 ± 0.05	1.1 ± 0.02	2.4 ± 0.05	2.2 ± 0.01
18:0	23.3 ± 0.6	28.2 ± 2.2	20.5 ± 0.1	21.0 ± 0.2	45.0 ± 1.4	45.0 ± 1.0	23.2 ± 0.8	23.7 ± 1.0
18:1	5.0 ± 0.1	5.0 ± 0.2	12.9 ± 0.05	12.7 ± 0.02	7.2 ± 0.9	7.4 ± 0.7	9.8 ± 0.3	9.8 ± 0.7
18:2	9.9 ± 0.3	10.1 ± 0.3	18.2 ± 0.2	18.9 ± 0.3	3.1 ± 0.1	2.9 ± 0.1	10.2 + 0.2	10.3 ± 0.2
20:3	0.1 + 0.0	0.1 ± 0.0	1.0 ± 0.05	1.0 ± 0.02	—	—	—	—
20:4	27.0 ± 1.7	26.9 ± 1.5	16.0 ± 0.1	15.8 ± 0.2	21.3 ± 1.2	20.5 ± 1.4	23.2 ± 1.0	22.9 ± 0.9
22:6	10.3 ± 0.3 [d]	9.0 ± 0.5	2.8 ± 0.1	3.1 ± 0.6	—	—	7.9 ± 0.9	5.4 ± 0.2
Unsaturation [b] index	195.2 ± 3.3 [e]	187.4 ± 2.8	136.7 ± 1.9	138.9 ± 1.6	99.7 ± 1.4	96.3 ± 1.8	172.8 ± 3.3	156.6 ± 3.5

[b] The unsaturation index is Σ mol fraction × number of unsaturated bonds in each acid.
[c] $P < 0.02$.
[d] $P < 0.01$.
[e] $P < 0.05$.

activity of untreated microsomes from diabetic rat liver showed transitions at two temperatures, which can be viewed as a transition extending between the two temperatures. In any event, the major difference in the activation energy profile of the enzyme activity of diabetic rats, as compared to that of normal rats, is the appearance of transition at higher temperatures suggesting a less fluid environment around the enzyme on the endoplasmic reticulum membrane. The important difference in the lipid composition is the increase of the percent molar ratio of cholesterol to phospholipid from 20 to 27 in whole microsomes and 8 to 14 in rough microsomes of alloxan-diabetic rat livers. The increased cholesterol:phospholipid ratio could significantly modify the thermotropic properties of the membrane. On the basis of studies of cholesterol-phosphatidylcholine mixed vesicles it has been concluded that increasing cholesterol content, in addition to its condensing effect, will increase the separation into cholesterol-rich and cholesterol-poor domains [28]. Rates of permeation [29] and enzyme activities [1] are also found to change with altered cholesterol concentration in liposomes. Moreover, cholesterol, in addition to causing a general decrease in membrane fluidity, is known to increase fluidity below the temperature of thermal transition and decrease fluidity above it [30]. Therefore, the appearance of transition at higher temperatures in the Arrhenius plot of the enzyme from diabetic rats may be partly due to a higher cholesterol to phospholipid ratio in the lipids of microsomes. The cholesterol effect is also evident in the incomplete modification of the trantion by 0.1% deoxycholate.

Substrate inhibition of glucose-6-phosphatase occurred at a lower glucose 6-phosphate concentration for the enzyme from diabetic rat liver (0.02—0.25 M) than for the enzyme of normal rat liver (0.06 M). The inhibition may not be due to increased ionic strength of the substrate in the medium since 10 mM $NaHSO_3$ did not show any inhibition [14]. The presence of 2.5 mM anilinonaphthalene sulfonate, which relieved the inhibition, did not abolish the transition in activation energy but only increased it to a higher temperature. Also, deoxycholate did not alter the inhibition or enthalpy of binding to the site but abolished the transition in activation energy of the enzyme of normal rats. Therefore, the inhibition phenomenon may be intrinsic to the enzyme and may not have significantly contributed to the transition in the energy of activation of the untreated enzyme. Since the transition in activation energy of the enzyme activity of normal rats is increased to a higher temperature, the presence of anilinonaphthalene sulfonate only decreases fluidity of the membrane lipids but the bilayer structure of microsomal vesicles is largely retained. At temperatures above the transition, because the lipid bilayer is fluid enough for conformational changes to be induced by anilinonaphthalene sulfonate, there is only one site for substrate binding with average affinity. Therefore, at high temperatures both V and apparent K_m values are increased yielding linear kinetics. At temperatures below the transition, the bilayer is less fluid and the two binding sites are distinctly maintained resulting in allosteric kinetics. Further, as a consequence of the increased binding of anilinonaphthalene sulfonate, particularly to protein domains at low temperatures, the sulfonate group can compete with the phosphate group of glucose 6-phosphate [15] for binding to the high affinity site, giving rise to competitive inhibition. However, other explanations for substrate inhibition and the anilinonaphthalene sulfonate

effect are also possible. For example, if the enzyme also functions as a glucose 6-phosphate transporter [7] at saturating substrate concentrations the bound transporter could hinder the phosphatase. Since phosphatase activity is rate-limiting, inhibition will occur, but could be relieved by the binding anilinonaphthalene sulfonate.

The influence of altered lipid environment of glucose-6-phosphatase from diabetic rat liver is reflected in its interaction with anilinonaphthalene sulfonate. Although the enzyme is activated at all temperatures, the allosteric effect exhibited by the enzyme of normal rats is absent at low temperatures. Because of the high cholesterol content of lipids, the bilayer may still be fluid enough for conformational changes to occur at low temperatures and, therefore, there is only one substrate binding site on the enzyme.

The significance of the absolute values of the thermodynamic parameters of activation of the glucose-6-phosphatase reaction should be viewed with caution. However, a comparison of values (Table I) derived under different experimental conditions should prove useful. The energy (or enthalpy) of activation is generally lower than that reported for acid or alkali catalyzed hydrolysis of glucose 6-phosphate [31]. The entropy of activation is large, but negative, suggesting a high orientation or order factor [31]. Also, transport or permeation of glucose 6-phosphate into microsomal vesicles may be another contributing factor [7]. Another noteworthy fact is that the free energy of activation (28—30 kcal/mol) is independent of temperature and other experimental conditions and is identical to that (approx. 29.0 kcal/mol) calculated from the data [31] for acid or alkaline hydrolysis of glucose 6-phosphate at 25.8°C. It appears, therefore, that similar energetic factors are operating in the formation of the transition state complex in enzymic and nonenzymic hydrolyses.

Glucose-6-phosphatase is, therefore, responsive not only to nutritional and hormonal stimuli [2] but also to physical and chemical perturbations of the bilayer structure of the endoplasmic reticulum membrane, as shown here. Alteration in the composition of lipids of the membranes during different pathophysiologic states could also alter the response of glucose-6-phosphatase to physical and chemical treatments. Dilantin has been found to be beneficial in the treatment of glycogen storage diseases Type I (Van Gierke) [32]. Furthermore, children on dilantin medication develop abnormal glucose tolerance [33]. Mechanism of dilantin action may be analogous to the glucose-6-phosphatase activation effect of anilinonaphthalene sulfonate as reported here.

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References

1 Lenaz, G., Curatola, G., Mazzanti, L. and Parenti-Castelli, G. (1978) Mol. Cell. Biochem. 22, 3—32
2 Ashmore, J. and Weber, G. (1959) Vitam. Horm. 17, 91—132
3 Hers, H.G., Berther, J., Berthet, L. and DeDure, C. (1951). Bull. Soc. Chim. Biol. 33, 21—27
4 Gunderson, H.M. and Nordlie, R.C. (1975) J. Biol. Chem. 250, 3552—3559
5 Nilsson, O.S. and Dallner, G. (1977) J. Cell. Biol. 72, 568—583

6 Nordlie, R.C. (1974) in Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.A., eds.), Vol. 8, pp. 33—117, Academic Press, New York
7 Nilsson, O.S., Anion, W.J., Depierre, J.W., Callner, G. and Ernster, L. (1978) Eur. J. Biochem. 82, 627—634
8 Duttera, S.M., Byrne, W.L. and Granoza, M.C. (1968) J. Biol. Chem. 243, 2216—2228
9 Zakim, D. (1970) J. Biol. Chem. 245, 4953—4961
10 Carland, R.C. and Cori, C.F. (1972)) Biochemistry 11, 4712—4718
11 Eletr, S., Zakim, D. and Vessey, D.A. (1973) J. Mol. Biol. 78, 351—362
12 Grinna, L.Y. (1975) Biochim. Biophys. Acta 403, 388—392
13 Silvius, J.R., Read, B.D. and McElhaney, R.N. (1978) Science 199, 902—904
14 Maddaiah, V.T., Kumbar, M. and Collipp, P.J. (1978) in Biomolecular Structure and Function (Agris, P.F., Loeppky, R.N. and Sykes, B.D., eds.), pp. 129—136, Academic Press, New York
15 Maddaiah, V.T., Chen, S.Y., Rezvani, I., Sharma, R. and Collipp, P.J. (1971) Biochem. Biophys. Res. Commun. 43, 114—118
16 Carvo, M., Maddaiah, V .T., Collipp, P.J. and Chen, S.Y. (1974) FEBS Lett. 39, 102—104
17 Dallner, G. (1974) Methods Enzymol. 31, 191—201
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Harris, R.A. (1971) Arch. Biochem. Biophys. 147, 436—445
20 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
21 Zlatkis, A. and Zak, B. (1969) Anal. Biochem. 29, 143—148
22 Parson, J. and Patton, S. (1967) J. Lipid Res. 8, 696—698
24 Arion, W.J., Wallin, B.K., Carlson, P.W. and Lange, A.L. (1972) J. Biol. Chem. 247, 2558—2565
25 Zierler, K. and Rogus, E. (1978) Biochim. Biophys. Acta 514, 37—53
26 Nordlie, R.C. and Arion, W.J. (1965) J. Biol. Chem. 240, 2155—2164
27 Fleischer, S. and Kervina, M. (1974) Methods Enzymol. 31, 3—41
28 Estep, T.N., Mountcastle, D.B., Biltonen, R.L. and Thompson, T.E., (1973) Biochemistry 18, 1984—1989
29 Clejan, S., Bittman, R., Deroo, P.W., Isaacson, Y.A. and Rosenthal, A.F. (1979) Biochemistry 18, 2118—2125
30 Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265—273
31 Bunton, C.A. and Chaimovich, H. (1966) J. Am. Chem. Soc. 88, 4082—4089
32 Jubiz, W. and Rallison, M.L. (1974) Arch. Intern. Med. 34, 418—421
33 Malherbe, C., Burrill, K.C., Levin, S.R., Karan, J.K. and Forshan, P.H. (1972) N. Engl. J. Med. 286, 339—342